Surface and Internal Galactosyl Receptors Are Heterooligomers and Retain This Structure after Ligand Internalization or Receptor Modulation†

Maryanne C. S. Herzig‡ and Paul H. Weigel*

Department of Human Biological Chemistry and Genetics, University of Texas Medical Branch, Galveston, Texas 77550

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ABSTRACT: We have developed a specific chemical affinity reagent for the hepatic galactosyl receptor (GalR) by derivatizing asialoorosomucoid (ASOR) with the homobifunctional *N*-hydroxysuccinimide (NHS) ester cross-linker disuccinimidyl suberate [Herzig, M. C. S., & Weigel, P. H. (1989) *Biochemistry 28*, 600]. NHS–ASOR cross-links with 30–50% efficiency to the three GalR subunits, designated rat hepatic lectins (RHL) 1, 2, and 3. Here, we examined the subunit structure of both surface and internal receptors of two functionally distinct GalR subpopulations, designated state 1 or state 2 GalR. Freshly isolated cells, referred to as state 1 cells, kept at 4 °C express only active state 1 GalR on their surface. When these cells are equilibrated at 37 °C, they then express both state 1 GalR and state 2 GalR on their surface. These cells are referred to as state 1,2 cells. After incubation at 4 °C with NHS–^{125}I-ASOR, surface or internal GalR of state 1 cells or of state 1,2 cells incorporated ^{125}I-ASOR into all three RHL subunits. As analyzed by autoradiography of SDS–PAGE, radiolabeling was identical for all conditions and was in a ratio of 1:1:1 for RHL 1:2:3. Native GalR structure was also examined by first cross-linking nonradiolabeled NHS–ASOR at 4 °C to surface or internal receptors of state 1 or state 1,2 hepatocytes. These cells were then washed with EGTA, extracted with Triton X-100, immunoprecipitated with anti-orosomucoid antibody, and subjected to Western blot analysis. Antisera specific for RHL 1 or RHL 2/3 detected cross-linked complexes of $M_r \simeq$ 85K or ≃90K–115K, respectively, as well as un-cross-linked native subunits. In all four cases, the ratio of free to cross-linked subunits was ≥5:1 for RHL 1 and ≤0.5:1 for RHL 2/3. Internalized GalR had the same ratio of free to cross-linked subunits as noninternalized GalR. Depletion of ATP either before or after cross-linking GalR to NHS/ASOR also did not alter the ratio of free to cross-linked RHL subunits. We conclude that the surface and internal GalR of the two functionally distinct GalR populations have the same heterooligomeric subunit composition and that this GalR structure persists following endocytosis or ATP depletion.

The mammalian hepatocyte galactosyl receptor (GalR)[1] binds specifically to and mediates endocytosis of triantennary glycoproteins whose oligosaccharide chains terminate with galactose or *N*-acetylgalactosamine (Ashwell & Harford, 1982). The GalR is approximately 264K in nonionic detergents (Andersen et al., 1982) with a binding domain of approximately 105K–148K (Steer et al., 1981) and has three subunits that are identified in SDS–PAGE. Designated RHL 1, 2, and 3, these subunits are of $M_r \simeq$41.5K, 49K, and 54K, respectively, and are the products of two different genes (Drickamer, 1987). RHL 1 is the predominant polypeptide of the GalR, while RHL 2 and 3 are minor components and differ only in the type and extent of posttranslational carbohydrate modification (Drickamer et al., 1984; Holland et al., 1984; Halberg et al., 1987). Products of both genes are required for expression of a GalR capable of endocytosing a natural triantennary glycoprotein ligand (McPhaul & Berg, 1986). However, Braiterman et al. (1988) reported that a highly galactosylated synthetic ligand is bound and endocytosed at a low level by transfected hepatoma tissue culture cells expressing only the RHL 1 polypeptide. Functional GalRs, with ligand binding capability, reside both on the surface and in the interior of hepatocytes at steady state (Weigel & Oka, 1983b; Baenziger & Fiete, 1980; Steer & Ashwell, 1980).

All three receptor subunits are capable of binding Gal residues (Lee & Lee, 1986; 1988; Halberg et al., 1987), yet there is no consensus on what comprises a receptor–ligand binding unit or the native receptor structure. In studies with a 3-Å cross-linker on hepatocyte microsomes, only homooligomeric cross-link products such as RHL 1-RHL 1 were detected (Halberg et al., 1987), suggesting that no physical association of RHL 1 and 2/3 exists. In other studies (Sawyer et al., 1988), antibodies to peptides specific for RHL 1 or RHL 2/3 were able to coimmunoprecipitate the other subunit, implying that heterooligomers do exist on the hepatocyte. The two nonidentical subunits of the human GalR were also reported to associate in HepG2 cells (Bischoff et al., 1988). Loss of both receptor subunits from the cell surface was induced by subunit-specific antibody, and heterooligomers were detected by chemical cross-linking on microsomal membranes.

The rat hepatic GalR system is further distinguished by having two subpopulations of functionally distinct GalR and two different parallel pathways for ligand uptake and degradation (Weigel et al., 1986; Oka & Weigel, 1987; Clarke et al., 1987). These pathways are modulated differently by temperature or by perturbing agents such as NaN_3, microtubule depolymerizing drugs, chloroquine, and monensin (McAbee et al., 1990). One of these subpopulations of GalR

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* To whom correspondence should be addressed.

‡ Present address: Department of Biochemistry, The University of Texas Health Science Center, Floyd Curl Dr., San Antonio, TX 78284.

[1] Abbreviations: GalR, galactosyl receptor; BSA, bovine serum albumin; RHL, rat hepatic lectin; ASOR, asialoorosomucoid; OR, orosomucoid; TBS, Tris-buffered saline; PBS, phosphate-buffered saline; NHS, *N*-hydroxysuccinimide; IgY, yolk immunoglobulin.

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constitutively recycles and is inactivated and then reactivated during transit through the endocytic pathway (McAbee & Weigel, 1987, 1988). Conceivably, different receptor structures, due to different RHL subunit compositions or ratios, could be the physical basis for these two functionally different receptor subpopulations and pathways.

In the present study, we used an affinity cross-linker for the GalR and antisera specific for RHL 1 or for RHL 2/3 to determine which subunits comprise a receptor–ligand complex and whether surface and intracellular receptors are structurally similar in the two functionally distinct GalR populations. We also examined the effects of internalization at 37 °C or ATP depletion on the structure of cross-linked GalR–ASOR complexes. We find that surface and internal GalRs have a similar heterooligomeric subunit composition and retain this association upon internalization. A preliminary report of these results appeared earlier (Herzig & Weigel, 1988).

Materials and Methods

Materials. Human orosomucoid (α_1-acid glycoprotein), from Sigma, was desialylated with neuraminidase to prepare ASOR, and ^{125}I-ASOR and ^{125}I protein A were prepared with Iodogen, from Pierce, as described previously (Weigel & Oka, 1982). Na^{125}I (10–20 mCi/μg of iodine) was from Amersham. Collagenase (type I), CNBr-activated Sepharose 4B, iodoacetamide, neuraminidase (type X), calf thymus DNA, ATP, Percoll, SDS, Nonidet P-40, Triton X-100, sodium deoxycholate, trypsin (type III from bovine pancreas), soybean trypsin inhibitor (type IIS), tris(hydroxymethyl)aminomethane (Tris), and 2-(*N*-morpholino)ethanesulfonic acid were from Sigma. Digitonin was from Kodak, BSA (clinical reagent grade, CRG-7) was from Armour Pharmaceutical Co., and protein A was from Genzyme. *N*-(2-Hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid was from Research Organics, Inc. Bisbenzimide (Hoechst dye 33258) was from Behring Diagnostics. *N*,*N*′-Methylenebis(acrylamide), ammonium persulfate, SDS–PAGE molecular weight standards, 4-chloro-1-naphthol, 5-bromo-4-chloro-3-indolyl phosphate, and nitro blue tetrazolium were from Bio-Rad. Acrylamide, twice recrystallized, was from U.S. Biochemical Corp. Dithiothreitol was from Boehringer Mannheim. Disuccinimidyl suberate and Bradford protein assay reagents were from Pierce. All other chemicals were reagent grade.

Media and Buffers. Medium 1 is a modified Eagle's medium (catalogue no. 420-1400) from Grand Island Biologicals Co. (GIBCO) supplemented with 2.4 g of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and 0.22 g/L $NaHCO_3$. Med 1/BSA is medium 1 with 0.1% (w/v) BSA, pH 7.4. Med 1/EGTA is medium 1 with 7.5 mM EGTA. Buffer 1 contains 143 mM NaCl, 6.8 mM KCl, and 10 mM HEPES, pH 7.4. BIC-10 is buffer 1 with 10 mM $CaCl_2$ while BIE-5 is buffer 1 with 5 mM EGTA. Hanks balanced salt solution was prepared according to the GIBCO formulation. PBS contains 137 mM NaCl, 8 mM sodium phosphate, 2.7 mM potassium chloride, and 1.5 mM potassium phosphate, pH 7.4. TBS contains 10 mM Tris-HCl and 154 mM NaCl, pH 7.4.

Antisera and Purified Antibodies. Antibodies to the affinity-purified GalR generated in goat were described previously (McAbee & Weigel 1988). RHL subunit-specific antisera raised in rabbit were a generous gift of K. Drickamer (Halberg et al., 1987). Antisera against human orosomucoid, which recognizes the desialylated protein ASOR, were a nephalometric-grade goat antisera or a rabbit IgG fraction from Sigma. Alkaline phosphatase conjugated to both rabbit anti-goat IgG (vs heavy and light chains) and goat anti-rabbit IgG (vs heavy and light chains) was from Bio-Rad. Rabbit anti-chicken IgG (vs the whole molecule) conjugated to horseradish peroxidase was from Sigma, and rabbit anti-chicken IgG (vs Fc) protein conjugated to alkaline phosphatase was from Jackson Labs. Antibody and protein A conjugation to CNBr-activated Sepharose 4B was as suggested by the manufacturer.

Antibodies to the GalR from rat (Ray & Weigel, 1985) were raised in white leghorn laying hens by multisite intramuscular injections with 100 μg of affinity-purified rat GalR mixed 1:1 with Freund's complete (primary inoculation) or incomplete adjuvant (secondary inoculation 1 month later). Eggs were harvested, and the yolks were separated from egg white, diluted 1:1 with PBS containing 0.05% sodium azide and 133 μM phenylmethanesulfonyl fluoride, and stored at −70 °C. Yolks were assayed for anti-GalR activity by a modified dot blot assay. Purified GalR in TBS was deposited on nitrocellulose paper (0.10 μg of GalR/well) and blocked with 10% milk solids (Carnation nonfat dry) in TBS. GalR spots, excised with a cork borer, were placed in 48-well plates for a 1-h incubation at room temperature with 400 μL of yolk dilutions. After being rinsed with TBS containing 0.05% NP40, spots were incubated at room temperature for 1 h with rabbit anti-chicken IgG (vs heavy and light chains) conjugated to horseradish peroxidase in TBS containing 5% BSA, washed, and developed with 4-chloro-1-naphthol and hydrogen peroxide (McAbee & Weigel, 1988).

IgYs from yolks with anti-GalR activity were isolated by a modification of the sequential poly(ethylene glycol) (PEG) precipitation procedure of Polson et al. (1980). Yolks were thawed, diluted with PBS to 300 mL at 4 °C, centrifuged at 25400*g* for 10 min at 4 °C, and filtered through 100-μm Nytex nylon cloth from Tetko. An initial incubation with 3.5% (w/v) PEG, MW = 8000, for 10 min at 24 °C precipitates lipids and vitellin. The supernatant recovered after centrifugation and filtration through 100-μm cloth was treated by addition of solid PEG to 12% (w/v) final concentration. After 10 min at 24 °C, the suspension was again centrifuged at 4 °C, and the pellets were redissolved in 300 mL of PBS for a second series of PEG precipitations. After centrifugation of the 4% PEG precipitation, the supernatants were filtered through 25-μm cloth, recentrifuged, filtered through bulk cotton, and reprecipitated by addition of PEG to 12%. Pellets obtained after centrifugation were recompacted by a final centrifugation, and the residual PEG was aspirated. Final pellets were resuspended in 10 mL of PBS, filtered through 0.22-μm filters, and assayed for protein. Approximately 80 mg of IgY was recovered per egg yolk.

Hepatocytes. Isolated hepatocytes from male Sprague Dawley rats were prepared by using a modification (Oka & Weigel, 1987) of the collagenase perfusion procedure of Seglen (1973). These freshly isolated cells, which are kept on ice after their preparation, express only state 1 GalR on their surfaces and are referred to as state 1 cells. Isolated cell suspensions incubated at 37 °C increase and stabilize their surface receptor content (Weigel & Oka, 1983a). These hepatocytes now express both state 1 and state 2 GalRs and are referred to as state 1,2 cells. All cell incubations at 37 °C were in a gyratory water bath shaking at 100 rpm ($\leq 4 \times 10^6$ cells/mL in Med 1/BSA, with cell suspensions occupying 10% of the flask volume).

Synthesis of NHS–ASOR. NHS esters of ASOR were prepared as described previously (Herzig & Weigel, 1989). A 500-fold molar excess of dissucinimidyl suberate (100 mg/mL in dimethyl sulfoxide) to ASOR amino groups was

reacted with ASOR (10–20 μM) in 0.1 M sodium borate, pH 8.0, for 2.5 min and brought to 50 mM 2-(*N*-morpholino)-ethanesulfonic acid (pH ≃5.0), and unreacted cross-linker was removed by centrifugation over a G-25 Sephadex column.

Cross-Linking NHS–ASOR to Receptor and Determination of Receptor Activity. Surface receptor activity was assessed by incubating suspensions of intact hepatocytes in BIC-10 with ^{125}I-ASOR or NHS–^{125}I-ASOR (≃1.5 μg/mL) at 4 °C for 60 min. Total cellular receptor activity was determined in the presence of 0.055% digitonin (Weigel et al., 1983). Internal receptors only were assessed after surface receptors had been occupied with nonradioactive ASOR at 9 μg/mL for 1 h. The cells were then washed 2 times with BIC-10 and treated with 0.055% digitonin before incubation and cross-linking with NHS–^{125}I-ASOR. Prior to determination of bound radioactivity, cells were washed twice by centrifugation in Hanks to determine total binding or in medium 1/EGTA or BIE-5 to remove non-cross-linked ASOR and determine cross-linked and nonspecifically bound material. Nonspecific binding was assessed by incubating cells with ligand in BIE-5 alone or in BIC-10 with a 100-fold excess of unlabeled ligand. Specific cross-linking is defined as the percent of the specific cell-associated radioactivity that is resistant to release by EGTA treatment.

Internalization of NHS–ASOR–GalR Complexes. State 1,2 cells were labeled and cross-linked with NHS–ASOR or NHS–^{125}I–ASOR for 1 h at 4 °C, washed twice with BIE-5, and resuspended in medium 1/BSA. Cells were incubated in a gyratory water bath at 37 °C, with one flask per time point. Cells were then chilled to 4 °C with 3 volumes of ice-cold Hanks before overlaying on discontinuous Percoll gradients composed of 0.5 mL of 50%, 10 mL of 40%, and 10 mL of 30% Percoll in BIE-5 in a 50-mL 28 × 112 mm polycarbonate round-bottom centrifuge tube (Clarke & Weigel, 1985). Gradients were centrifuged at 450*g* for 10 min at 4 °C. Only intact cells (>98% viable) isolated from the bottom of the 40% layer were analyzed further. In some instances, covalently bound ASOR on the cell surface was removed with trypsin at 6 mg/mL in medium 1/EGTA for 30 min at 10 °C, after which 2 mg/mg soybean trypsin inhibitor was added and the cells were washed once in medium 1/EGTA. ^{125}I radioactivity was determined if appropriate, or cells were extracted in 1% Triton X-100 in BIE-5.

ATP Depletion. Cellular ATP levels were reduced with sodium azide treatment either before or after ligand binding. Two different methods of azide treatment were employed, both of which deplete the intracellular ATP concentration by ≥98%. (i) Cells were incubated on ice in 10 mM NaN_3 and 2 mM NaF in medium 1/BSA for 10 min and then for 10 min at 15 °C in a gyratory shaker (Clarke & Weigel, 1985). This depletes ATP without causing a concomitant redistribution of surface receptors to the cell interior. (ii) Alternatively, cells were treated at 37 °C with 15 mM NaN_3 in medium 1/BSA for 30 min and then rapidly chilled by addition of 3–4 volumes of ice-cold Hanks containing 10 mM NaN_3 (McAbee & Weigel, 1988). This causes depletion of ATP, with a redistribution of surface receptor internally. After azide treatment, all samples were overlaid on discontinuous Percoll gradients containing 10 mM NaN_3. The intact viable cells were recovered from the Percoll gradients for subsequent ligand binding or immediate cell extraction. Cells pretreated with azide were recovered from Percoll gradients, washed and resuspended in BIC-10, and allowed to bind NHS–ASOR to surface and/or internal receptors as described above. Post-treated cells were first cross-linked with NHS-ASOR for 1 h at 4 °C, washed twice with BIE-5, and resuspended in medium 1/BSA for their subsequent azide treatment. Ultimately, all samples were extracted with 1% Triton X-100 in BIE-5 containing 10 mM NaN_3 and then immunoprecipitated with goat anti-OR Sepharose prior to SDS–PAGE and Western blot analysis.

Cell Extractions and Immunoprecipitations. Cells (8 × 10^6/mL) in either buffer 1, BIC-10, or BIE-5, as indicated, and containing 133 mM phenylmethanesulfonyl fluoride and 1% Triton X-100, were rotated at 4 °C for ≥20 min. Alternatively, cell samples without Triton X-100 were sonicated 90 s in a bath sonicator. After centrifugation at 4 °C at 12000*g* in a Beckman J2-21 centrifuge for 20 min or for 10 min in a Beckman Microfuge at top speed, the extracts were analyzed for protein, immunoprecipitated, and/or subjected to SDS–PAGE. Cell extracts in 1% Triton X-100 BIE-5 were immunoprecipitated using either goat or rabbit anti-human OR antibodies conjugated to CNBr-activated Sepharose 4B (1.75 mg of antibody/mL of resin) at a final concentration of ≃2% (v/v) antibody–Sepharose. After 1 h at 4 °C, samples were centrifuged and the pellets washed twice in 1.25 mL of BIE-5 containing 0.05% Triton X-100. Alternatively, in a two-step immunoprecipitation, cell extracts were diluted to 0.5% Triton X-100 with BIC-10 or BIE-5, as indicated, and incubated by rotation with antibody for 1 h at 4 °C prior to addition of protein A–Sepharose to a final concentration of ≃5% (v/v) for a second hour of incubation. Samples were then washed as above with BIC-10 or BIE-5 containing 0.05% Triton X-100.

Electrophoresis. SDS–PAGE was performed as described by Laemmli (1970) on both cell extracts and immunoprecipitated samples diluted with a 4-fold concentrated sample buffer of 0.25 M Tris-HCl, pH 6.8, 8% (w/v) SDS, 40% (v/v) glycerol, and 0.04% (w/v) bromphenol blue. Reduced samples were treated with 11 mM dithiothreitol, boiled for 7–10 min, and then alkylated with 50 mM iodoacetamide. The samples were resolved by electrophoresis on 6.5% or 8.0% (w/v) acrylamide slab gels, of 10 × 15 cm (Idea Scientific mini-slab STILTS apparatus) or 14 × 16 cm (Hoefer Vertical Slab apparatus). After electrophoresis, gels stained with Coomassie Blue R-250 were dried between two porous cellophane sheets (Hoefer) and exposed to KODAK X-OMAT AR film at −70 °C either with or without a phosphor screen (Kodak X-Omatic 1533-6051).

Western Blotting. Western blotting was by the method of Burnette (1981) with some buffer modifications (McAbee & Weigel, 1988). Transfer of proteins from SDS–PAGE to nitrocellulose paper was in an Idea Scientific GENIE electrophoretic blotter. The protein blots were analyzed immediately at room temperature or after storage at 4 °C. Nonspecific binding was blocked by incubation for 1 h in TBS containing 5% (w/v) BSA, 10% (w/v) nonfat milk solids (Carnation), and 0.2% (w/v) Nonidet P-40. Antisera specific for RHL 1 or RHL 2/3 were recovered after each primary incubation for reuse. These primary antisera incubations were for 12 h using a 1:200 dilution. Chicken anti-GalR and rabbit anti-OR were incubated at dilutions of 1:1000 and 1:300, respectively. After being washed, the nitrocellulose strips were incubated with 5 mg/mL ^{125}I protein A (≃9 × 10^6 cpm/mL) in TBS containing 5% BSA for 90 min, and then washed, dried, and autoradiographed. Alternatively, detection was with alkaline phosphatase conjugated secondary antibodies directed against rabbit (or goat) heavy and light IgG chains. This secondary antibody incubation was for 90 min at a 1:1000 dilution, following which immunoblots were washed and de-

veloped with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium at 225 and 450 μg/mL in 0.1 M $NaHCO_3$/1 mM $MgCl_2$, pH 9.6, as recommended by Bio-Rad. When the desired level of staining was obtained, the nitrocellulose paper was thoroughly washed with water and air-dried. Densitometric scans (Helena Laboratories, Quick Scan Jr.) were performed on X-ray film exposed without the phosphor screen or preflashed with a Xenon flash unit (Vivitar 125). Analysis of the densitometry scans was done only in the region of linear response. In some instances, Polaroid negatives (type 55 film) of the Western blots developed with alkaline phosphatase conjugated antibodies were also analyzed by densitometry.

General. Cellular DNA was determined by the method of Labarca and Paigen (1980) using calf thymus DNA as the standard. Protein was determined by the methods of Bradford (1976) or Lowry et al. (1951) with BSA as the standard. A luciferin–luciferase procedure employing a Beckman LS 7500 scintillation counter for photon detection was used to quantitate ATP (Weigel & Englund, 1975). Centrifugations of intact cells were at 80*g* for 3 min in a Beckman Model TJ-6 refrigerated table-top centrifuge. Samples prior to immunoprecipitation or electrophoresis were centrifuged for 10 s in a Beckman Model B microfuge. ^{125}I radioactivity was determined on a Packard Multiprias 2 γ spectrometer.

Results

NHS–^{125}I-ASOR Cross-Linking to Surface and Internal GalRs of State 1 and State 1,2 Hepatocytes. Rat hepatocytes have steady-state populations of both surface and internal GalRs. Additionally, two surface populations of active GalR exist with respect to their modulation by temperature, microtubule drugs, chloroquine, monensin, vanadate, or depletion of ATP (Weigel & Oka, 1983a; Oka & Weigel, 1989; McAbee et al., 1990). Freshly isolated cells express only one population of GalR on their surface, designated state 1 GalR. We refer to these cells as state 1 cells. This GalR population is insensitive to modulation by the above agents. Cells incubated at 37 °C for 1 h increase their surface GalR activity by at least 2-fold. These additional, recruited receptors, which we term state 2 GalRs, constitutively recycle and are modulated by these various inhibitors. We refer to these 37 °C equilibrated cells as state 1,2 cells. In previous affinity cross-linking studies, we examined the surface GalR on state 1,2 cells and found that all three subunits cross-link to NHS–ASOR in a ratio of 1:1:1, implying that all three subunits are involved somehow in the native surface GalR (Herzig & Weigel, 1989).

Here we addressed whether the functional difference seen between state 1 and state 2 GalR populations are reflected by a different pattern of radiolabeled NHS–ASOR cross-linking. Surface or internal GalRs of state 1 or state 1,2 hepatocytes were allowed to cross-link to NHS–^{125}I-ASOR. Samples were washed with EGTA to remove non-cross-linked material and then extracted in Triton X-100 and subjected to SDS–PAGE and autoradiography (Figure 1). Identical autoradiographic patterns are seen for all conditions of labeling, although intensities of labeling varied. Bands from 80K to 104K correspond to cross-linked complexes between NHS–^{125}I-ASOR (41K) and the 42K RHL 1 (ASOR–RHL 1, $M_r \simeq 83\,000$), the 54K RHL 2 (ASOR–RHL 2, $M_r \simeq 95\,000$), and the 64K RHL 3, (ASOR–RHL 3, $M_r \simeq 105\,000$). The ratio of incorporation of radiolabel into RHL 1:2:3 was ≃1:1:1 for all GalR situations examined (Table I) with no apparent preference for cross-linking to any one subunit over another. Surface and internal GalRs from both state 1 and state 1,2 cells appear to have a similar composition and cannot be distinguished by a distinctive pattern of NHS–ASOR–RHL cross-linking.

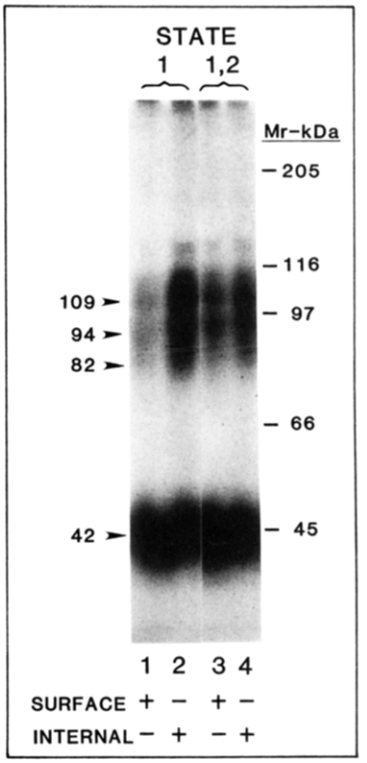

FIGURE 1: NHS–^{125}I-ASOR cross-linking to surface or intracellular GalR. NHS–^{125}I-ASOR was incubated with state 1 (lanes 1 and 2) or state 1,2 (lanes 3 and 4) cells at 4 °C and allowed to cross-link to GalR subunits as described under Materials and Methods. Surface receptors were labeled directly (lanes 1 and 3). Internal receptors were accessed with digitonin and labeled with NHS–^{125}I-ASOR after an initial prebinding of surface receptors with unlabeled ASOR (lanes 2 and 4). Cells were then washed with EGTA, and after sonication in BIE-5, equal amounts of protein were subjected to reduced SDS–PAGE and autoradiography. Samples were brought to equal protein content by addition of nonradiolabeled cell extract. Radiolabeled cross-linking products at $M_r \simeq 84K$, 93K, and 105K correspond to cross-links between ASOR and RHL 1, 2, and 3, respectively. Molecular weight determinations were made from coelectrophoresed standards depicted on the right. State 1 cells specifically bound 207 fmol of NHS–^{125}I-ASOR/10^6 cells on their surface and specifically cross-linked 23 fmol. State 1,2 cells specifically bound 303 fmol of NHS–^{125}I-ASOR/10^6 cells and specifically cross-linked 34.5 fmol.

Table I: Incorporation of NHS–^{125}I-ASOR into the Three GalR Subunits[a]

		Gal receptor subunit		
	condition	RHL 1	RHL 2	RHL 3
state 1	surface[b]	1.00 ● 0.05	1.13 ± 0.04	1.08 ● 0.05
state 1	internal[b]	1.00 ● 0.05	1.06 ● 0.07	0.92 ± 0.07
state 1,2	surface[c]	1.00 ± 0.13	1.10 ● 0.13	1.00 ± 0.10
state 1,2	internal[c]	1.00 ± 0.05	1.14 ± 0.01	0.98 ● 0.06

[a] NHS–^{125}I-ASOR was cross-linked at 4 °C to hepatocytes as described under Materials and Methods. The cells were washed with EGTA, extracted, and subjected to reduced SDS–PAGE, and the gels were dried and autoradiographed. The data are presented as a relative ratio of radiolabeled ASOR incorporation into RHL 1, 2, and 3 with radioactive incorporation into RHL 1 arbitrarily set to 1.00. Each ratio (±SEM) was determined from three separate cell binding experiments. [b] Samples were quantitatively immunoprecipitated by anti-OR–Sepharose. The gel portions corresponding to the three main autoradiographic bands were excised, and ^{125}I radioactivity was determined directly. [c] Cross-linked cell extracts were subjected to SDS–PAGE, and densitometry scans of autoradiograms were analyzed.

Detection of NHS–ASOR–GalR Complexes by Western Blotting. Antisera specific for RHL 1 or RHL 2/3 were a generous gift from K. Drickamer (Halberg et al., 1987). The

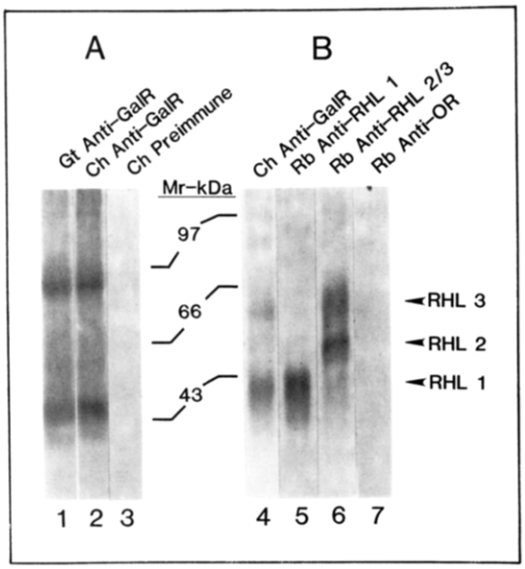

FIGURE 2: Subunit specificity of anti-RHL antibodies. GalR, affinity-purified on ASOR–Sepharose, was subjected to nonreducing SDS–PAGE and transferred to nitrocellulose. Two separate preparations of GalR, showing different extents of RHL subunit dimer formation were run on gels A and B. Western blot analysis was done using as primary antisera anti-GalR IgG from goat (lane 1), anti-GalR IgY from chicken (lanes 2 and 4), preimmune IgY from chicken (lane 3), rabbit antiserum to RHL 1 (lane 5), rabbit antiserum to RHL 2/3 (lane 6), and goat antiserum to human OR (lane 7). The secondary incubation was with alkaline phosphatase conjugated to anti-species antibody followed by development with nitro blue tetrazolium dye as described under Materials and Methods. Coelectrophoresed standards are depicted in the center; locations of RHL bands are shown at the right.

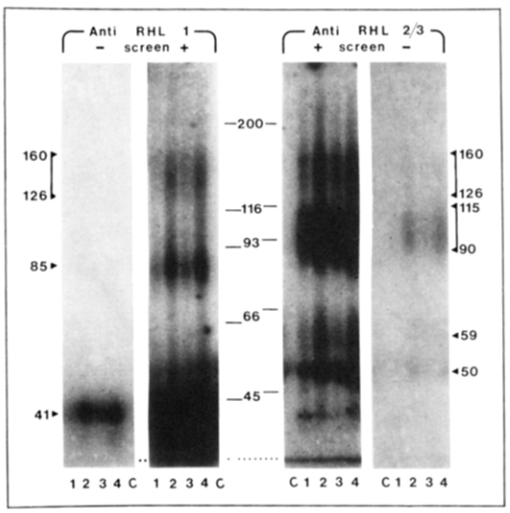

FIGURE 3: Western blot analyses of RHL–ASOR complexes following immunoprecipitation with anti-OR antibody. State 1 (lanes 1 and 2) and state 1,2 (lanes 3 and 4) hepatocytes were cross-linked with nonradioactive NHS–ASOR both on the surface (lanes 1 and 3) and intracellularly (lanes 2 and 4) as described in Figure 1 and under Materials and Methods. State 1,2 hepatocytes permeabilized with digitonin were incubated with ASOR as a control (lane C). All samples were then washed with EGTA, extracted with 1% Triton X-100 in BIE-5, immunoprecipitated with rabbit anti-OR–Sepharose, and subjected to reduced SDS–PAGE followed by Western blot analysis. Rabbit anti-RHL 1 or anti-RHL 2/3 antiserum was the primary reagent, and ^{125}I protein A was the detecting agent. Autoradiography was for 10 days either with or without a phosphor screen and preflashed film to give two different exposures. Molecular weights ($\times 10^{-3}$) of coelectrophoresed standards are depicted in the middle of the figure. Free RHLs 1, 2, and 3 are indicated by arrowheads at M_r ~41K, 50K, and 59K, respectively.

specificity of these antisera for their respective subunits in purified GalR was confirmed, whereas anti-OR antibodies were nonreactive with the GalR (Figure 2). We then used these reagents to verify that ASOR was indeed cross-linked to each subunit. Both the surface and internal GalRs on state 1 and state 1,2 hepatocytes were cross-linked to nonradiolabeled NHS–ASOR. State 1,2 cells were surface-labeled with nonderivatized ASOR as a control. After Triton X-100 extraction in BIE-5, samples were immunoprecipitated with rabbit anti-OR–Sepharose.

The ASOR–receptor cross-linking gives a unique way to examine the components that comprise a ligand–receptor complex, since the anti-OR immunoprecipitation isolates only ASOR, ASOR cross-linked to GalR subunits, and proteins associated with the cross-linked subunits. By inference, proteins immunoprecipitated with anti-OR–Sepharose are also in the receptor complex that binds and cross-links to ASOR. These immunoprecipitated samples were then subjected to SDS–PAGE followed by immunoblotting with the specific antisera for RHL 1 or 2/3 and detection with ^{125}I protein A (Figure 3). No autoradiographic development was seen in the ASOR control. Any original ASOR–receptor complexes had been dissociated by the EGTA, and therefore, no free RHL subunits were coprecipitated by the anti-OR–Sepharose. Apparent development at longer exposures was due to spillover from adjacent lanes. In cross-linked GalR samples, the same autoradiographic patterns existed in all conditions despite the known functional differences between the state 1 and state 2 GalR populations. Anti-RHL 1 detected a band of ≃85K, and anti-RHL 2/3 detected a region from 90K to 105K. Thus, NHS–ASOR does cross-link to all three subunits of surface GalR in both GalR populations. Multimeric cross-linked products of ≃120K were visualized by both antisera. It is unclear whether these are the result of two RHL subunits cross-linking to one ASOR or vice versa.

Most interestingly, free un-cross-linked RHL subunits exist in a complex with cross-linked NHS–ASOR–RHL subunits. Free RHL 1, 2, and 3 subunits (indicated by arrowheads at 41K, 50K, and 59K in Figure 3) were coimmunoprecipitated with the cross-linked ASOR–RHL. The ratio of free subunits to cross-linked subunits differed greatly for RHL 1 vs RHL 2/3 (Table II). Free RHL 1 was the predominant species detected with anti-RHL 1, while cross-linked RHL 2/3–ASOR itself was the predominant species detected by anti-RHL 2/3.

Verification of ASOR–RHL 1 Cross-Linked Complexes. Individual RHL subunits of affinity-purified GalR can form dimers, which are seen even in reduced SDS–PAGE (Harford et al., 1982). When monomeric subunits, isolated by elution from polyacrylamide gels, are reexamined on SDS–PAGE, both monomers and dimers are then found (Drickamer et al., 1984). Underivatized ^{125}I-ASOR also shows some evidence of dimer formation, as 1–2% of the iodinated preparation is found at 80K on SDS–PAGE (unpublished result). These two observations raise the possibility that the putative cross-linked RHL 1–ASOR complex identified by Western blot analysis is actually an RHL 1 dimer comigrating with an ASOR dimer and that RHL 1 itself is not cross-linked to ligand. To assess this possibility, hepatocytes incubated with NHS–^{125}I-ASOR were denatured by boiling in SDS-containing buffer before immunoprecipitation with anti-OR–Sepharose. Boiling followed by a 50-fold dilution did not interfere with immunoprecipitation of ^{125}I-ASOR (not shown). If the 82K species detected by the anti-RHL 1 antiserum is actually an RHL 1 homodimer merely associated with authentic cross-linked GalR–ASOR, then denaturation prior to immunoprecipitation should obliterate staining in this region of the Western blots.

Table II: Immunoblot and Densitometry Analysis of Gal Receptors[a]

ligand	cellular experimental condition	receptor location	antibody specificity	amount of free vs cross-linked RHL subunits (% of total radioactivity) RHL 1	RHL 2/3	ASOR–RHL 1	ASOR–RHL 2/3	ASOR–RHL multimer
NHS–ASOR	state 1	surface	RHL 1	84.2	0	7.9	0	7.8
			RHL 2/3	0	14.2	0	72.8	13.0
NHS–ASOR	state 1	internal	RHL 1	73.6	0	17.5	0	8.9
			RHL 2/3	0	26.2	0	52.3	21.4
NHS–ASOR	state 1,2	surface	RHL 1	76.6	0	12.5	0	11.0
			RHL 2/3	0	20.5	0	61.9	17.5
NHS–ASOR	state 1,2	internal	RHL 1	68.4	0	19.8	0	11.8
			RHL 2/3	0	23.1	0	59.0	17.9
ASOR	state 1,2	total	RHL 1	0	0	0	0	0
			RHL 2/3	0	0	0	0	0

[a] Autoradiograms from Figure 3 were analyzed by densitometry. The data are presented as the percent of the total radioactivity per lane found in each radioactive band in the SDS–PAGE gel.

This is clearly not the case (Figure 4). These cell extracts had RHL 1–ASOR complexes as detected with both RHL 1 specific antisera (lane 4, panel B) and GalR-specific antisera (lane 4, panel A). Denaturation did occur, as no free subunits were coprecipitated with the ASOR–RHL cross-linked complexes. Samples not denatured but diluted 50-fold prior to immunoprecipitation showed both free RHL subunits and also cross-linked RHL–ASOR subunits (lanes 3). Thus, we conclude that the 82K band is indeed an ASOR–RHL 1 cross-link and not homocomplexes of either ASOR–ASOR or RHL 1–RHL 1.

Effect of Internalization on GalR Structure. Although the rat hepatic GalR internalizes ligand, which then becomes targeted for degradation, the receptor itself recycles back to the surface. Recently, McAbee and Weigel (1988) proposed that during recycling of the state 2 GalR, an intracellular inactivation/reactivation cycle occurs, wherein the receptor is transiently unable to bind ligand. We have proposed that the GalR is a heterooligomer composed of six RHL subunits with three subunits participating in the binding of a triantennary ligand (Herzig & Weigel, 1989). It is conceivable that the GalR inactivation process involves a dissociation or rearrangement of RHL subunits after internalization. Therefore, we used the NHS–ASOR affinity cross-linker to analyze the structure of GalR from state 1,2 cells before and after endocytosis at 37 °C (Figure 5). Cross-linked NHS–ASOR–GalR on control cells incubated at 4 °C was removed almost completely by trypsin treatment (Figure 5A, lane 2). However, in cells incubated at 37 °C for 7.5 min, the majority of these cross-linked complexes was resistant to trypsin due to the internalization of the surface GalR (Figure 5A, lane 3). After covalent NHS–ASOR–GalR complexes were internalized, the ratios of free to cross-linked GalR subunits detected by RHL-specific antisera in analysis of cell extracts did not differ from cells remaining at 4 °C (Figure 5, panel B). Even cells incubated at 37 °C for 30 min showed no change in the Western blot pattern of ASOR–RHL complexes or free RHL subunits. Therefore, GalR internalization does not lead to a redistribution of RHL subunits in these cross-linked receptor–ASOR complexes.

Effect of ATP Depletion on GalR Structure. Sodium azide treatment of hepatocytes depletes ATP levels, and the method of ATP depletion can affect the distribution of GalR between the cell surface and interior. Treatment at 37 °C for 30 min with 5–15 mM NaN_3 results in ATP depletion and an inactivation of state 2 GalRs as well as a redistribution of these GalRs from the surface to the interior of the hepatocyte (McAbee & Weigel, 1989). Hepatocytes treated with 10 mM azide and 2 mM NaF at 15 °C also have depleted ATP levels, yet retain their surface GalR activity (Clarke & Weigel, 1985).

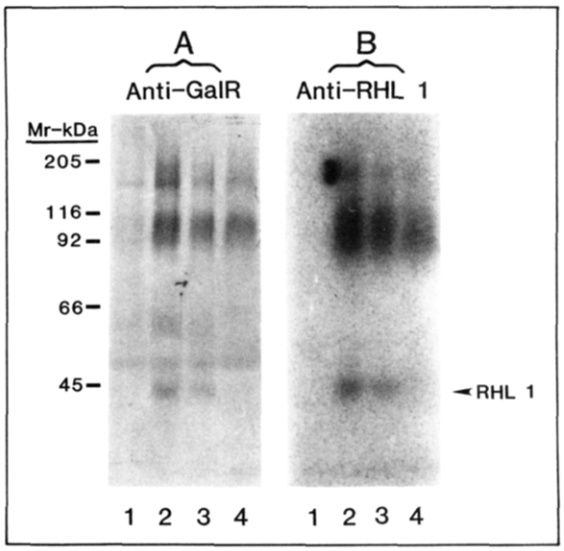

FIGURE 4: Immunoprecipitation and Western blot analysis of denatured cross-linked GalR. State 1,2 hepatocytes were chilled on ice, placed in 0.055% digitonin in BIC-10, and allowed to bind and cross-link ^{125}I-ASOR (lanes 1) or NHS–^{125}I-ASOR (lanes 2–4). Cells were washed with EGTA, extracted in 2% Triton X-100 containing BIE-5, and then diluted either 4-fold (lanes 1 and 2) or 50-fold (lanes 3) with 0.5% Triton X-100/BIE-5 or were denatured by dilution with 4× Laemmli (1970) buffer and boiling for 5 min before a 50-fold dilution in 0.5% Triton X-100/BIE-5 (lanes 4). All samples were immunoprecipitated by incubation with 100 μL of a 50% suspension of rabbit anti-OR–Sepharose, subjected to reduced SDS–PAGE using equal cell protein/sample, and transferred to nitrocellulose for Western blot analysis. Nitrocellulose was probed with chicken anti-GalR and detected with rabbit anti-chicken IgG–alkaline phosphatase (panel A) or with rabbit anti-RHL 1 and ^{125}I protein A (panel B). Coelectrophoresed molecular weight standards are depicted to the left.

Therefore, we examined the effects of these two different azide treatments on the GalR complex (Figure 6). Azide treatment after ligand binding was used to examine the effect of ATP depletion on already formed NHS–ASOR–GalR complexes. Azide treatment before ligand binding was used to examine the effect of ATP depletion on residual active GalR complexes. In each case, cells were washed with EGTA after binding and cross-linking to NHS–ASOR in order to leave only the cross-linked and nonspecifically bound ligand on the cell surface. Following azide treatment, only viable cells were further analyzed.

Control cells bound with ASOR showed no autoradiographic development in the region of ASOR–GalR cross-links, and no free RHL subunits were present (Figure 6A–C, lanes d). NHS–ASOR cross-linked to state 1 or state 1,2 hepatocytes that were not ATP-depleted showed cross-linked GalR–ASOR complexes at $M_r \simeq$80K–108K (lanes e, panels A–C) and free

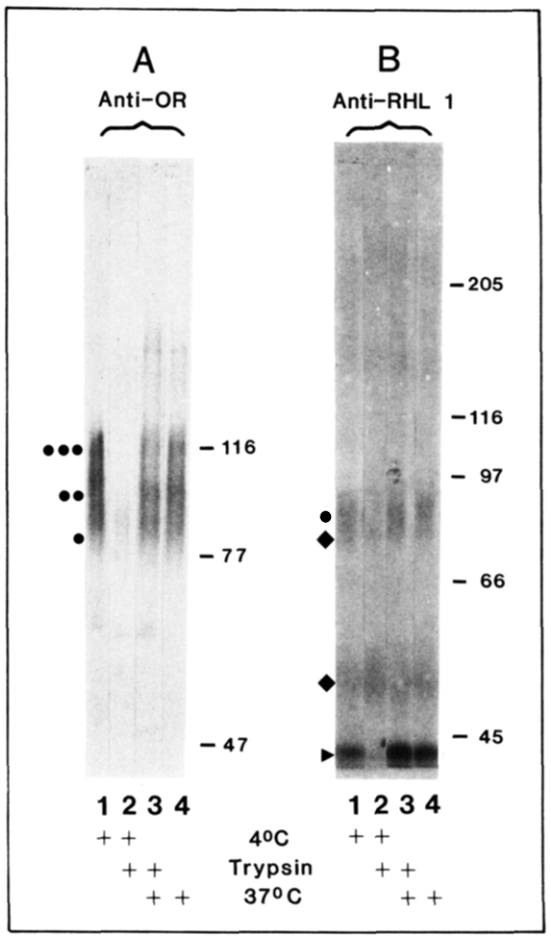

FIGURE 5: Effect of internalization on the structure of cross-linked NHS-ASOR-GalR complexes. State 1,2 hepatocytes were allowed to bind and cross-link to NHS-ASOR at 4 °C, washed with EGTA, and resuspended in medium 1/BSA. Cells were then incubated at 4 °C (lanes 1 and 2) or at 37 °C for 7.5 (lanes 3) or 30 (lanes 4) min. Viable cells were isolated from Percoll gradients. Residual surface GalR-ASOR complexes were destroyed with trypsin as described under Materials and Methods (lanes 2 and 3). Cell samples were then extracted in BIE-5/1% Triton X-100 and immunoprecipitated with goat anti-OR-Sepharose. Equivalent cell samples were subjected to SDS-PAGE and electroblotted onto nitrocellulose. Panel A was probed with rabbit anti-OR, and panel B was probed with rabbit anti-RHL 1. Detection was by incubation with goat anti-rabbit IgG conjugated to alkaline phosphatase. Coelectrophoresed standards are depicted to the right. Diamonds (♦) mark the location of antibody bands, while cross-linked ASOR-RHL subunit complexes are marked by one, two, or three dots (●) for RHL 1, 2, or 3, respectively. The location of free RHL 1 is shown by an arrowhead.

RHL subunits (lanes e, panels B and C). Free RHL 1 is the predominant GalR polypeptide detected. The intensities of staining differ, which reflects the modulation (loss) of active GalR on the surface by both low temperature and azide treatment. This pattern of NHS-ASOR-GalR complex staining in Western blot analysis of control cells proved invariant for all the treatments tested. ATP depletion and concomitant inactivation of state 2 GalR by a 37 °C azide treatment (Figure 6, lanes k and l) gave results identical with ATP depletion at 15 °C (lane j). Pretreatment of cells with azide before NHS-ASOR cross-linking (lanes k–j) gave results indistinguishable from cells depleted of ATP after ligand cross-linking (lanes a, b, f, g). Western blot analysis of state 1 cells (lanes a–c) and state 1,2 cells (lanes e–l) shows identical patterns. This invariant pattern is independent of the detection method.

Figure 7 shows Western blot analysis using ^{125}I protein A of state 1,2 hepatocytes pre- and posttreated with azide. Again, although the intensities of autoradiographic development differed, the patterns of development are the same. Densitometric scans of the autoradiograms showed that ratios of free to cross-linked RHL subunit were invariant regardless of cell treatment (not shown). In three separate experiments examining both state 1 and state 1,2 hepatocytes, no rearrangement of GalR complexes was observed when either protocol for ATP depletion was used before or after ligand binding. Therefore, some mechanism other than subunit dissociation and redistribution or reorganization appears responsible for state 2 GalR inactivation and reactivation during recycling.

Rabbit Anti-RHL 1 or -RHL 2/3 Coimmunoprecipitates All RHL Subunits. The presence of dissimilar receptor subunits after immunoprecipitation with anti-OR-Sepharose does not prove that these subunits are associated. An NHS-ASOR-GalR complex could be composed of like RHL subunits. An equal number of RHL 1, RHL 2, and RHL 3 homooligomeric complexes that were cross-linked to ASOR would result in the 1:1:1 radiolabel incorporation summarized in Table I. Therefore, we determined whether the native, non-cross-linked GalR in Triton X-100 extracts of isolated hepatocytes is composed of like or unlike subunits by performing immunoprecipitations of cell extracts with rabbit anti-RHL 1 or anti-RHL 2/3 followed by Western blot analysis of these immunoprecipitates with the same antibodies (Figure 8). Since both the analogous chicken hepatic receptor (Loeb & Drickamer, 1988) and the rat GalR (Andersen et al., 1982) display conformational or aggregation state changes in the presence and absence of Ca^{2+}, we performed the extractions and immunoprecipitations in either 10 mM $CaCl_2$ or 5 mM EGTA. The autoradiograms of the Western blots clearly show coimmunoprecipitation of free RHL subunits in agreement with the recent report of Sawyer et al. (1988). The amount of RHL 2/3 immunoprecipitated by anti-RHL 1 (Figure 8, lanes 6 and 8) was less than that precipitated by anti-RHL 2/3 (Figure 8, lanes 5 and 7). RHL 1 was equally precipitated by either anti-RHL or anti-RHL 2/3 (Figure 8, lanes 1–4). Therefore, different RHL subunits are associated in the same GalR complex both in the presence and in the absence of Ca^{2+}. This is consistent with the above conclusion that cross-linked NHS-ASOR-GalR complexes, immunoprecipitated by anti-OR-Sepharose, are also composed of unlike subunits.

Discussion

One of the most persistent problems in our understanding of the GalR is knowing what comprises a native GalR capable of binding and internalizing triantennary ligands. The mole ratios reported for the three subunits of the affinity-purified rat GalR identified on SDS-PAGE vary from 2.5:1:1 to 8:1:1 for RHL 1 to RHL 2 to RHL 3 (Ashwell & Harford, 1982; Drickamer et al., 1984; Lee & Lee, 1986; Takahashi et al., 1985). By gel filtration, the size of the GalR is ≃264K (Andersen et al., 1982), and since each RHL subunit has at most two monosaccharide binding sites (Lee & Lee, 1988), a native GalR for a triantennary ligand must be composed of more than one subunit. Therefore, current research centers on determining whether the native GalR is homooligomeric or heterooligomeric. Two lines of evidence support a homooligomeric model. Although RHL 1 is the major subunit, the predominant species radiolabeled by lactoperoxidase iodination of the cell surface is RHL 2/3 (Warren & Doyle, 1981; Schwartz et al., 1981; Lee & Lee, 1987). Lack of RHL 1 labeling indicates that RHL 1 may be localized apart from the labeled RHL 2 and might mean that each subunit type

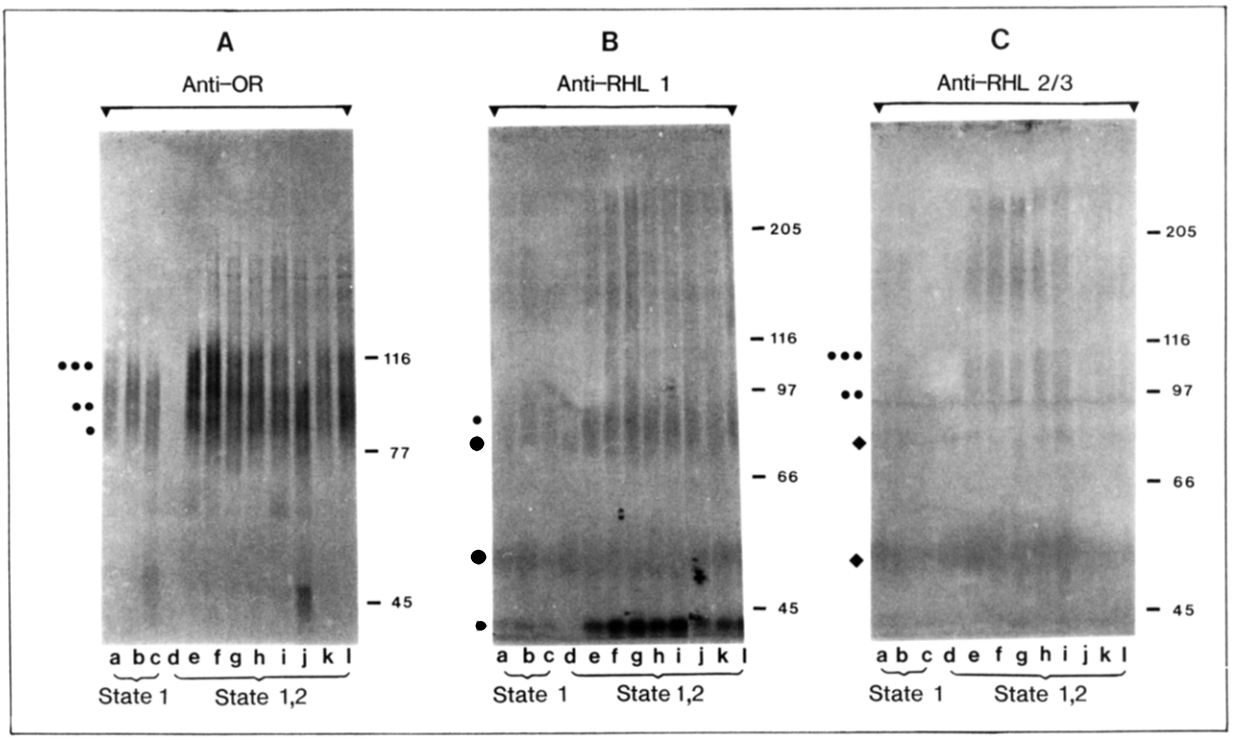

FIGURE 6: Effect of cellular ATP depletion on GalR structure. Freshly isolated hepatocytes expressing state 1 GalR on their surface (lanes a–c) or hepatocytes incubated at 37 °C for 1 h and expressing both state 1 and state 2 GalR on their surface (lanes d–l) were used. Azide pretreatment before NHS–ASOR cross-linking was at 10 °C with NaN_3/NaF (lanes c and j) or at 37 °C with NaN_3 alone (lanes k and l) as described under Materials and Methods. After azide treatment, viable cells were isolated from Percoll gradients, resuspended in BIC-10, and allowed to bind NHS–ASOR to surface (lanes c, j, and k) or total cellular receptors (lanes l) as described under Materials and Methods. Cells first allowed to bind ASOR (lanes d) or NHS–ASOR (lanes a, b, and e–i) were washed with EGTA and replaced in medium 1/BSA. As controls, cells with bound ASOR (lanes d) or NHS–ASOR (lanes a and e) remained on ice at 4 °C or were allowed to internalize GalR–ASOR complexes at 37 °C for 7.5 min (lanes i) or 30 min (lanes h). After the 7.5-min internalization, residual surface receptors were removed with trypsin. Hepatocytes containing bound NHS–ASOR were posttreated with azide at 10 °C (lanes f) or placed at 37 °C with 10 mM NaN_3 for 30 min (lanes b and g). Viable cells were isolated by Percoll gradients. All cell samples were extracted in BIE-5 containing 1% Triton X-100, immunoprecipitated with goat anti-OR–Sepharose, and subjected to SDS–PAGE and Western blot analysis. Panel A was probed with rabbit anti-OR, panel B with rabbit anti-RHL 1, and panel C with rabbit anti-RHL 2/3. Detection was with alkaline phosphatase conjugated secondary antibodies. Coelectrophoresed standards are depicted to the right. Diamonds (♦) mark the location of antibody bands. ASOR–RHL conjugates are marked by one, two, or three dots (●) for RHL 1, 2, or 3, respectively. The location of free RHL 1 is shown by an arrowhead. Panel A appears to show some differences between samples pretreated with NaN_3/NaF at 10 °C and control cells. However, this resulted from background staining in the immunoblot. When a second Western blot with anti-OR was performed on these samples, no differences were detected in the cross-linked region (not shown).

is in a homooligomer. Preferential surface iodination, however, may also be due to a readily accessible Tyr at the extracellular C-termini of RHL 2/3 subunits (Halberg et al., 1987) as opposed to a preponderance of these subunits at the cell surface. Additionally, Halberg et al. (1987) concluded that RHLs 1, 2, and 3 are self-associated into separate GalRs, each having Gal binding activity, based on studies with the cross-linking agent difluorodinitrobenzene on rat liver microsomes in a Ca^{2+}-deficient buffer. They suggested that different RHL homooligomers may be active at separate stages during the endocytic process.

On the other hand, evidence continues to accumulate for a heterooligomeric model. Several studies have demonstrated the importance of RHL 1 association with RHL 2/3 for GalR activity. McPhaul and Berg (1986) found that both RHL 1 and RHL 2/3 gene products were required for expression of a functional GalR capable of binding ASOR in transfected cells. Braiterman et al. (1989) isolated a transfected clone expressing only RHL 1 that was capable of binding and internalizing a synthetic polygalactosylated ligand but could not bind or process a natural ligand, ASOR. Graeve et al. (1988, 1990) reported that in MDCK cells transfected with only one cDNA, RHL 1 remains in the perinuclear region with a half-life of ≤3 h, while RHL 2/3 is localized on the basolateral surface and has a half-life of 12–15 h. In doubly transfected cells, all subunits were expressed at the cell surface and had half-lives of ≥12 h. In stably transfected murine NIH-3T3 cells, expression of both subunits of the human GalR is also required for ASOR binding (Shia & Lodish, 1989).

Furthermore, the conclusion that the GalR is a heterooligomeric complex, composed of unlike subunits, is supported by the coimmunoprecipitation data presented here and is in agreement with the work of Bischoff et al. (1988) and Sawyer et al. (1988). The latter researchers demonstrated that their RHL-specific antipeptide antisera would not detect the other RHL subunit(s). Yet, all three RHL subunits of the GalR were coprecipitated by either specific antipeptide antibody. We also find that immunoprecipitation of one subunit by its specific antisera immunoprecipitates the other RHL subunits. RHL 1 is the predominant component of either the anti-RHL 1 or the anti-RHL 2/3 immunoprecipitates from detergent extracts of hepatocytes. Our interpretation of these results is that all RHL 2/3 subunits are in a complex with RHL 1, since RHL 1 is equally precipitated by anti-RHL 1 or anti-RHL 2/3. However, this then means that not all RHL 1 is present in a complex with RHL 2/3, since the quantity of RHL 2/3 immunoprecipitated by anti-RHL 1 is less than that immunoprecipitated by anti-RHL 2/3.

The reasons for the discrepancies with the cross-linking data of Halberg et al. (1987) are not clear. Perhaps the RHL

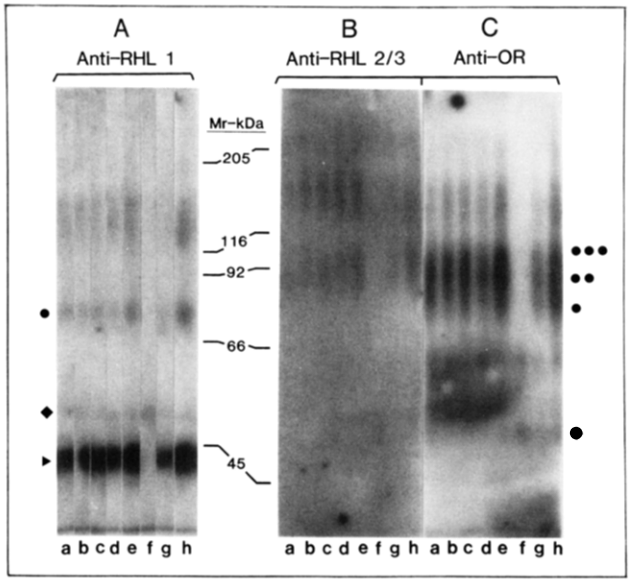

FIGURE 7: Effect of ATP depletion on GalR structure: detection by ^{125}I protein A. State 1,2, hepatocytes were pretreated at 15 °C for 10 min with NaN_3/NaF (lanes e) or at 37 °C for 30 min with NaN_3 alone (lanes g and h). After azide treatment, viable cells were isolated from Percoll gradients, resuspended in BIC-10, and allowed to bind NHS–ASOR to surface (lanes e and g) or internal GalRs (lanes h) as described under Materials and Methods. State 1,2 hepatocytes first allowed to bind NHS–ASOR (lanes a–d) or ASOR (lanes f) at 4 °C for 1 h, were washed with EGTA and replaced in Med 1/BSA. They were then kept at 4 °C as controls (lanes a and f) or were incubated at 37 °C with (lanes b) and without (lanes c) NaN_3 or were incubated at 15 °C (lanes d) with NaN_3/NaF. All cell samples were extracted in BIE 5 containing 1% Triton X-100, immunoprecipitated with goat anti-OR–Sepharose, and subjected to SDS–PAGE, transfer to nitrocellulose, and Western blot analysis. Panel A was probed with rabbit anti-RHL 1, panel B with rabbit anti-RHL 2/3, and panel C with anti-OR. Detection was with ^{125}I protein A and autoradiography. Positions of coelectrophoresed standards are shown between panels A and B. Diamonds (♦) mark the location of antibody bands detected in this system while ASOR–RHL cross-links are marked by one, two, or three dots (●) for RHL 1, 2, or 3, respectively. The location of free RHL 1 is shown by an arrowhead.

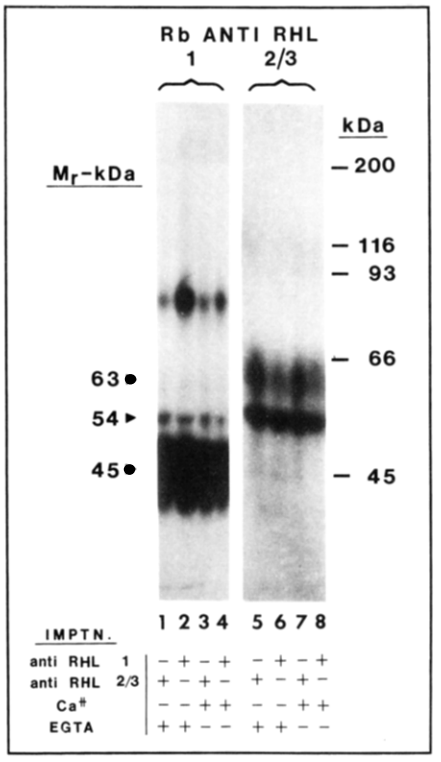

FIGURE 8: RHL subunit coimmunoprecipitation by RHL subunit-specific antisera. State 1,2 hepatocytes extracted in buffer 1 containing 1% Triton X-100 were immunoprecipitated with rabbit anti-RHL 1 (lanes 2, 4, 6, and 8) or anti-RHL 2/3 (lanes 1, 3, 5, and 7) in either BIC-10 (lanes 3, 4, 7, and 8) or BIE-5 (lanes 1, 2, 5, and 6). Following reduced SDS–PAGE and transfer to nitrocellulose, the blot was probed with either rabbit anti-RHL 1 (lanes 1–4) or rabbit anti-RHL 2/3 (lanes 5–8) followed by ^{125}I protein A and autoradiography. Coelectrophoresed standards are depicted to the right.

homooligomers they observed resulted from the use of the very short (3 Å) cross-linker. Cross-linking in the present study was done under ligand binding conditions (with Ca^{2+} present and the ligand bound) and with a cross-linker that has an arm of ≃11.4 Å attached to a 40K protein. Our results do not preclude the presence of some homooligomers. An unlikely possibility is that homooligomers of RHL 1 could associate with homooligomers of RHL 2/3 and result in the same immunoprecipitation data found by us and Sawyer et al. (1988). The native GalR would then approach 750K, and these sizes, however, are not seen in detergent extracts (Andersen et al., 1982; Nakada & Tashiro, 1983; Kawasaki & Ashwell, 1976). The results from this and the other studies discussed above are consistent with the conclusion that most RHL 1 and virtually all RHL 2/3 are present as a functional heterooligomeric GalR. These data also indicate the likely possibility that "extra" RHL 1 may be present as a minimally functional homooligomeric species. This could explain, in part, the different results observed by us and Halberg et al. (1987).

It was possible that the homooligomeric vs heterooligomeric GalR models could be reconciled with our evidence for the presence of GalR subpopulations with functional differences. Rat hepatocytes display two functionally different populations of GalR that are active in separate parallel pathways of receptor-mediated endocytosis (Weigel, 1987). These two pathways are distinguished by the response of their surface receptors to modulation by low temperature, metabolic energy poisons, microtubule drugs, monensin, chloroquine, and vanadate (Weigel & Oka, 1983a; Oka & Weigel, 1989; McAbee et al., 1990). State 2 GalR activity is lost in response to these agents, whereas the state 1 GalR population shows no change. A simple partitioning of the three different RHL subunits between surface and internal GalR populations or between the state 1 and state 2 GalR populations could reconcile the above data and provide a structural explanation for these functional differences.

If certain RHL subunits are restricted to the cell surface or cell interior, or to one GalR pathway over the other, then one would expect enrichment of NHS–ASOR cross-linking to one RHL subunit over another. The ratio of free to cross-linked RHL subunits, seen by Western blotting of receptor–ligand complexes, would also be different. However, the results with NHS–^{125}I-ASOR show identical cross-linking patterns for GalR on state 1 or state 1,2 cells, or on the cell surface or interior. In all cases, the three RHL subunits are in close enough proximity to bound NHS–ASOR to be cross-linked. No different arrangement of RHL subunits was detected. However, any GalR structural differences due to minor subunit rearrangement or posttranslational modifications, such as phosphorylation, acetylation, or desialylation, are beyond the detection limits of this approach.

We next examined the structural behavior of the GalR as it functions during endocytosis. After surface GalRs internalize ASOR, there are two rate constants for dissociation of the intracellular GalR–ASOR complexes (Oka & Weigel, 1983; Harford et al., 1983). State 2 GalR–ASOR complexes dissociate rapidly ($k \simeq 0.28$ min^{-1}), while state 1 GalR–ASOR complexes dissociate much slower ($k \simeq 0.014$ min^{-1}). Low

endosomal pH is presumed to cause this dissociation (Tycko et al., 1983; Goldstein et al., 1985; Wileman et al., 1985), although the rapid dissociation occurs before GalR–ASOR complexes reach endosomes of pH $\simeq$5.3 (Schmid et al., 1989). One can envision that the mechanism that drives receptor–ligand dissociation is the dissolution of a complex of RHL subunits. However, we detected no dissociation or rearrangement of RHL subunits after internalization. The ratios of free to cross-linked RHL subunits are the same for GalR–ASOR complexes that remain on the cell surface as those that are internalized at 37 °C. Although the covalent cross-link to ASOR might alter the normal state of the GalR after endocytosis, there is no evidence that internalization per se affects receptor subunit organization.

Similarly, we examined the native GalR structure after depletion of cellular ATP. Intracellular accumulation of inactive GalRs occurs when ATP is depleted (McAbee & Weigel, 1987, 1988). This is due to an intracellular reversible transient inactivation/reactivation cycle that state 2 GalRs undergo during constitutive recycling. Again, receptor subunit dissociation or reorganization could be responsible for this inactivation, with subunit reassociation being responsible for reactivation. GalRs were examined under two conditions of ATP depletion: one which causes GalR inactivation and one which does not. Cross-linking to NHS–ASOR was performed either before or after ATP depletion. No dissociation or reassociation of any RHL subunits could be discerned in the GalR–ASOR cross-linked complexes. Loeb and Drickamer (1987) followed the cross-linking pattern of the chicken hepatic lectin and also concluded that the oligomeric state of this receptor was unchanged during recycling.

We previously suggested that the rat GalR is a heterohexamer composed of four RHL 1 subunits and two subunits of RHL 2/3 (Herzig & Weigel, 1989). Three receptor isotypes are then possible: $(\text{RHL 1})_4(\text{RHL 2})_2$, $(\text{RHL 1})_4(\text{RHL 3})_2$, and $(\text{RHL 1})_4(\text{RHL 2})(\text{RHL 3})$. As each subunit is capable of binding one or two sugar residues (Lee & Lee, 1988; Halberg et al., 1987), a triantennary oligosaccharide binding site would be composed of a trimer of three subunits with their sugar binding domains located on the vertices of a right triangle with sides of 15, 22, and 25 Å (Lee et al., 1984). The predicted triantennary ligand binding domain, located extracellularly, is $\sim$148K in size, in agreement with the radiation inactivation study of Steer et al. (1981).

The present study shows for the first time that both surface and internal active GalRs are identical in their subunit structure. The functional differences seen between state 1 and state 2 GalR are not due to differences in the RHL subunit composition of these receptors. The GalR apparently keeps the same subunit organization throughout its cellular itinerary and its functional lifetime.

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Registry No. 5′-ATP, 56-65-5; galactose, 59-23-4.

References

Andersen, T. T., Freytag, J. W., & Hill, R. L. (1982) *J. Biol. Chem. 257*, 8036–8041.

Ashwell, G., & Harford, J. (1982) *Annu. Rev. Biochem. 51*, 531–554.

Baenziger, J. U., & Fiete, D. (1980) *Cell 22*, 611–620.

Bischoff, J., Libresco, S., Shia, M. A., & Lodish, H. F. (1988) *J. Cell Biol. 106*, 1067–1074.

Bradford, M. (1976) *Anal. Biochem. 72*, 248–254.

Braiterman, L. T., Chance, S. C., Porter, W. R., Lee, Y. C., Townsend, R. R., & Hubbard, A. L. (1989) *J. Biol. Chem. 264*, 1682–1688.

Burnette, W. N. (1981) *Anal. Biochem. 112*, 195–203.

Clarke, B. L., & Weigel, P. H. (1985) *J. Biol. Chem. 260*, 128–133.

Clarke, B. L., Oka, J. A., & Weigel, P. H. (1987) *J. Biol. Chem. 262*, 17384–17392.

Drickamer, K. (1987) *Kidney Int. 32*, S167–S180.

Drickamer, K., Mamon, J. F., Binns, G., & Leung, J. O. (1984) *J. Biol. Chem. 259*, 770–778.

Goldstein, J. L., Brown, M. S., Anderson, R. G. N., Russell, D. W., & Schneider, W. J. (1985) *Annu. Rev. Cell Biol. 1*, 1–39.

Graeve, L., Patzak, A., Drickamer, K., & Rodriguez-Boulan, E. (1988) *J. Cell Biol. 107*, 769a.

Graeve, L., Patzak, A., Drickamer, K., & Rodriguez-Boulan, E. (1990) *J. Biol. Chem. 265*, 1216–1224.

Halberg, D. F., Wager, R. E., Farrell, D. C., Hildreth, J., IV, Quesenberry, M. S., Loeb, J. A., Holland, E. C., & Drickamer, K. (1987) *J. Biol. Chem. 262*, 9828–9838.

Harford, J., Lowe, M., Tsunoo, H., & Ashwell, G. (1982) *J. Biol. Chem. 257*, 12685–12690.

Harford, J., Bridges, D., Ashwell, G., & Klausner, R. D. (1983) *J. Biol. Chem. 258*, 3191–3197.

Herzig, M. C. S., & Weigel, P. H. (1988) *J. Cell Biol. 107*, 769.

Herzig, M. C. S., & Weigel, P. H. (1989) *Biochemistry 28*, 600–610.

Holland, E. C., Leung, J., & Drickamer, K. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 7338–7342.

Kawasaki, T., & Ashwell, G. (1976) *J. Biol. Chem. 251*, 1296–1302.

Labarca, C., & Paigen, K. (1980) *Anal. Biochem. 102*, 344–352.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Lee, R. T., & Lee, Y. C. (1986) *Biochemistry 25*, 6835–6841.

Lee, R. T., & Lee, Y. C. (1987) *Biochemistry 26*, 6320–6329.

Lee, R. T., & Lee, Y. C. (1988) *Biochem. Biophys. Res Commun. 155*, 1444–1451.

Lee, R. T., Lin, P., & Lee, Y. C. (1984) *Biochemistry 23*, 4255–4261.

Loeb, A. A., & Drickamer, K. (1987) *J. Biol. Chem. 262*, 3022–3029.

Loeb, A. A., & Drickamer, K. (1988) *J. Biol. Chem. 263*, 9752–9760.

Lowry, O. H., Roseborough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

McAbee, D. D., & Weigel, P. H. (1987) *J. Biol. Chem. 262*, 1942–1945.

McAbee, D. D., & Weigel, P. H. (1988) *Biochemistry 27*, 2061–2069.

McAbee, D. D., Clarke, B. L., Oka, J. A., & Weigel, P. H. (1990) *J. Biol. Chem. 265*, 629–635.

McPhaul, M., & Berg, P. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 8863–8867.

Nakada, H., & Tashiro, Y. (1983) *J. Biochem. 94*, 1689–1695.

Oka, J. A., & Weigel, P. H. (1983) *J. Biol. Chem. 258*, 10253–10262.

Oka, J. A., & Weigel, P. H. (1987) *J. Cell. Physiol. 133*, 243–252.

Oka, J. A., & Weigel, P. H. (1989) *J. Cell Biol. 109*, 139.
Polson, A., von Wechmar, M. B., & van Regenmortel, M. H. V. (1980) *Immunol. Commun. 9*, 475–493.
Ray, D. A., & Weigel, P. H. (1985) *Anal. Biochem. 145*, 37–46.
Sawyer, J. T., Sanford, J. P., & Doyle, D. (1988) *J. Biol. Chem. 263*, 10534–10538.
Schmid, S., Fuchs, R., Kielian, M., Helenius, A., & Mellman, I. (1989) *J. Cell Biol. 108*, 1291–1300.
Schwartz, A. L., Marshak-Rothstein, A., Rup, D., & Lodish, H. F. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 3348–3352.
Seglen, P. O. (1973) *Exp. Cell Res. 82*, 391–398.
Shia, M. A., & Lodish, H. F. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 1158–1162.
Steer, C. J., & Ashwell, G. (1980) *J. Biol. Chem. 255*, 3008–3013.
Steer, C. J., Kempner, E. S., & Ashwell, G. (1981) *J. Biol. Chem. 256*, 5851–5856.
Takahashi, T., Nakada, H., Okumura, T., Sawamura, T., & Tashiro, Y. (1985) *Biochem. Biophys. Res. Commun. 126*, 1054–1060.
Tycko, B., Keith, C. H., & Maxfield, F. R. (1983) *J. Cell Biol. 97*, 1762–1776.
Warren, R., & Doyle, D. (1981) *J. Biol. Chem. 256*, 1346–1355.
Weigel, P. H. (1987) in *Vertebrate Lectins* (Olden, K., & Parent, B., Eds.) pp 65–91, Van Nostrand–Reinhold, New York.
Weigel, P. H., & Englund, P. T. (1975) *J. Biol. Chem. 250*, 8536–8542.
Weigel, P. H., & Oka, J. A. (1982) *J. Biol. Chem. 257*, 1201–1207.
Weigel, P. H., & Oka, J. A. (1983a) *J. Biol. Chem. 258*, 5089–5094.
Weigel, P. H., & Oka, J. A. (1983b) *J. Biol. Chem. 258*, 5095–5102.
Weigel, P. H., Ray, D. A., & Oka, J. A. (1983) *Anal. Biochem. 133*, 437–449.
Weigel, P. H., Clarke, B. L., & Oka, J. A. (1986) *Biochem. Biophys. Res. Commun. 140*, 43–50.
Wileman, T., Boshans, R., & Stahl, P. (1985) *J. Biol. Chem. 260*, 7387–7393.

Effects of Ions and pH on the Thermal Stability of Thin and Thick Filaments of Skeletal Muscle: High-Sensitivity Differential Scanning Calorimetric Study†

Antonio Bertazzon and Tian Yow Tsong*
Department of Biochemistry, University of Minnesota College of Biological Sciences, St. Paul, Minnesota 55108
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ABSTRACT: Differential scanning calorimetry (DSC) is unique for studying conformational changes in supramolecular structures because it is immune to interference by the turbidity and other optical artifacts of a sample solution. We have employed DSC to study thermal stability of myosin and actin in their filamentous forms (i.e., thick and thin filaments). The thermal stability of the myosin monomer, as well as polymers, showed remarkable sensitivities to pH and to the ionic strength of the solution. At pH 7.5, the endotherm of myosin filaments was broad and resembled that of the monomer in solution. Reducing the pH to 6.3 split the endotherm of the filament into two major transitions. The first one, with a T_m of 47 °C, a ΔH_{cal} of 805 kcal/mol, and a cooperative ratio (CR) of 0.1, was relatively insensitive to the pH changes whereas the second one which represented approximately 80% of the helical structure was pH sensitive. The second transition released 2.17 H^+ per mole at 0.17 M KCl and was defined by a T_m of 53.9 °C, a ΔH_{cal} of 917 kcal/mol, and a CR of 0.35. The major fragment contributing to the splitting of the endotherm was interpreted to be S-2 because the T_m of purified S-2 in a similar medium also shifted from 39.5 °C at pH 7.3 to 49.6 °C at pH 6.0. KCl had similar effects on the shape of the endotherm of the thick filament. A decrease of KCl from 0.2 to 0.1 M enhanced the effect of pH on the second transition. A calculated release of 8.9 K^+ per mole was associated with the melting of the major part of the helix. The T_m vs pH curve had an inflection point at pH 6.8 in 0.17 M KCl. These conditions mimicked the physiological conditions. The two major transitions of myosin filaments did not show a strong cooperativity (CR 0.1 and 0.4, respectively), implying that the domains observed in the monomer [Bertazzon, A., & Tsong, T. Y. (1989) *Biochemistry 28*, 9784–9790] were also present in the filament. In actin, a partial polymerization was observed when the concentration of Ca^{2+} in the medium was increased (1–10 mM), as assessed by analysis of the DSC endotherms (the CR increased from 0.7 to 1.25). This effect of calcium was not observed in the fully polymerized protein; however, a reduction of the pH from 7.9 to 5.9 increased the T_m from 68.2 to 74.3 °C, with apparently no effect on the cooperative ratio. These results suggest that, in contrast to the myosin filament, the thin filament behaved as a fully cooperative structure. The effect of pH on the thermal stability of F-actin was consistent with the release of 1.4 H^+ per mole upon melting.

The thermal stability of most components of the sarcomere has been studied in detail in solution. However, the aggregation of myosin to form filaments precludes any physical study based on spectroscopy or viscometry. By monitoring the change in solution pH as a function of temperature, Goodno and Swenson (1975a,b) and Goodno et al. (1976) were able to circumvent the problem to a certain degree. The melting of the whole myosin and of the light meromyosin (LMM) was shown to be characterized by the absorption of protons at 41 °C. Melting of S-2, on the contrary, was accompanied by a

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